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LARGE-SCALE FRACTIONATION OF S-FORM LIPOPOLYSACCHARIDE FROM *SALMONELLA ABORTUS EQUI*

CHEMICAL AND SEROLOGICAL CHARACTERIZATION OF THE FRACTIONS

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SUMMARY

The S-form lipopolysaccharide of *Salmonella abortus equi* was separated by a newly elaborated extraction method with organic solvents into three fractions of different chain length of the O-polysaccharide they contained. The three fractions were designated long-chain (20–50 repeating units), short-chain (0–6) and R-fraction (no repeating units) according to their migration pattern in polyacrylamide gel electrophoresis in the presence of sodium dodecylsulphate. The nature of the fractions as long- and short-chain and as R-fraction was confirmed by chemical analysis. The concentration of O-specific sugars was highest in the long-chain fraction, where their molar ratio to glucosamine was *ca.* 25:1. In the short-chain fraction the ratio of O-sugars to glucosamine was 2.5:1, and in the R-fraction O-specific sugars were absent. The serological properties of the three fractions were in good agreement with their chemical composition.

INTRODUCTION

Lipopolysaccharide (LPS) represents an essential constituent of the outer membrane of the gram-negative bacterial cell. It functions as the main heat-stable antigen, the O-antigen, and is at the same time the endotoxin of these bacteria[1,2]. As such, LPS is an important factor in pathogenicity, being responsible for many pathophysiological effects occurring in gram-negative infection. LPS are constructed according to a common general principle[3]. They consist of a polysaccharide region containing the O-specific chain and the core, and a lipid, the so-called lipid A. The O-chain is built up of repeating units of oligosaccharides. In contrast to LPS from wild-type S- (smooth) form bacteria, LPS from mutant (LPS-detective) R- (rough) form bacteria lack the O-chain, thus containing only core, or fragments of it, and lipid A. Since

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lipid A represents the biologically active centre of LPS, defective R-form LPS also represent highly active endotoxins[4].

LPS derived from S-form bacteria exhibit a high degree of heterogeneity, which is caused by the presence of molecules with different length of O-chains, *i.e.* with different numbers of repeating units[5–8], which may range from zero (R-form LPS) to 50. This heterogeneity becomes evident when LPS are analysed by polyacrylamide gel electrophoresis (PAGE), in the presence of sodium dodecylsulphate (SDS). In this way S-form preparations are resolved into numerous bands with a ladder-like pattern.

The migration distances of the various bands are inversely related to their molecular masses and reflect the number of repeating units present in the O-chain of the different fractions. All LPS analysed so far by SDS-PAGE have been found to contain R-form material, and therefore the biological behaviour of S-form preparations as studied so far represents mainly the combined effect of both LPS classes.

S- and R-form LPS are known to exhibit differences in their biological behaviour even though their toxic activities are very similar. One such difference is concerned with their organ distribution *in vivo*. Thus whereas the hepatic uptake of S-form LPS from the blood is effected exclusively by sinusoidal (Kupffer) cells, that of the R-form is effected both by Kupffer cells and hepatocytes, *i.e.* in contrast to S-form, R-form LPS has direct access to hepatocytes[9]. Another important difference exists in the interaction of S- and R-form LPS with high density lipoprotein *in vivo*, the result of which is that large amounts of S-form LPS accumulate in the adrenal glands whereas only insignificant amounts of R-form LPS are found in this organ[10]. S- and R-form LPS also show large differences in their ability to induce chemiluminescence in granulocytes[11], R-form LPS being very potent whereas S-form LPS are virtually inactive. Finally, S- and R-forms show distinct qualitative differences in their interactions with the complement system[12].

It seemed important therefore to resolve S-form LPS into fractions of S-form and R-form LPS. This would enable chemical and biological studies on genuine S-form LPS and on the R-form LPS synthesized by S-form bacteria. This paper reports the separation of S-form LPS from *Salmonella abortus equi* into fractions containing long-chain, short-chain, and R-form (no chain) LPS. The chemical compositions and serological properties of the three fractions are investigated and compared with those of the starting LPS.

EXPERIMENTAL

Lipopolysaccharides

LPS of *S. abortus equi* was isolated by the phenol–water method and subsequently purified by the phenol–chloroform–light petroleum (b.p. 40–60°C) procedure, and by ultracentrifugation[13]. Finally the LPS was electrodialysed and converted into its triethylamine salt form[13].

Sugar and fatty acid analysis

Neutral sugars were measured as their alditol acetate derivatives by gas–liquid chromatography (GLC) (Varian 1400), after hydrolysis of LPS in 0.1 *M* hydrochloric acid at 100°C for 48 h[14]. Xylose was used as internal standard. Fatty acids were

measured as methyl esters by GLC[15] in a Varian gas–liquid chromatograph (Model 3700) equipped with a flame ionization detector connected to a Hewlett-Packard integrator (Model 3380). 3-Hydroxydodecanoic and heptadecanoic acids were used as internal standards.

Colorimetric methods

Total phosphorus and glucosamine were measured according to refs. 16 and 17, respectively. 2-Keto-3-deoxy-D-manno-octonate (KDO) was determined by the thiobarbituric acid method as modified by Karkhanis *et al.*[18]. Abequose was estimated by the method of Cynkin and Ashwell[19].

Sodium dodecylsulphate-polyacrylamide gel electrophoresis

SDS-PAGE analysis of LPS and fractionated materials was performed in a discontinuous buffer system with a 5% stacking and a separating gel, according to Laemmli[7]. Gel size and thickness were 20 × 20 × 0.15 cm. A 2-μg preparation in 5 μl was loaded in each well. The current was 15 mA for stacking, and 20 mA for separating; LPS was detected by silver staining[8].

Serological assay

The antigenic properties of the three fractions obtained from *S. abortus equi* LPS were investigated by the passive haemolysis and passive haemolysis-inhibition test as described previously[20]. The three fractions were tested in relation to their ability to interact with anti-*S. abortus equi* O-antibodies and with antibodies to the various R classes (Ra–Re). The antisera used were raised in rabbits immunized with the corresponding heat-killed bacteria as described previously[20].

RESULTS

Fractionation of the lipopolysaccharide

The LPS was suspended in solvent A (chloroform–methanol–0.2 *M* hydrochloric acid, 138:127:41.5 v/v; 40 ml/g LPS) and stirred at 4°C for 15 min. After centrifugation (600 *g*, 10 min) a lower phase containing short-chain and R-fraction, and a small upper phase containing insoluble long-chain fraction were obtained. The two phases were separated, neutralized with triethylamine and evaporated. The solid materials were redissolved in water, electrodialysed, and freeze-dried.

The crude long-chain fraction was purified by repeated extraction with solvent A (two or three times).

The crude short-chain fraction, which contained R-LPS, was further fractionated by extraction with solvent B [chloroform–methanol–light petroleum–8 *M* hydrochloric acid, 12.5:4:4.5:0.2 (v/v)]. After centrifugation as above, the lower phase was enriched in R-LPS, the upper in short-chain LPS. Both fractions were isolated and then subjected to extraction with solvent C [chloroform–methanol–light petroleum–8 *M* hydrochloric acid, 10:4:2.5:0.25 (v/v)]. The procedure for isolation of the fractions was as above. In this way, pure R-fraction and short-chain fraction (still contaminated with R-LPS) were obtained.

The three fractions were designated long-chain (yield 30% based on the original LPS), short-chain (50%), and R-fraction (10%).

TABLE I

CONSTITUENTS OF *S. ABORTUS EQUI* LIPOPOLYSACCHARIDE AND THE SUBFRACTIONS

All preparations contain about three glucosamine residues (one in the core, two in lipid A).

Constituent	*Long-chain fraction*		*Short-chain fraction*		*R-fraction*		*Original lipopolysaccharide*	
	nmol/mg	*mol/GlcN*	*nmol/mg*	*mol/GlcN*	*nmol/mg*	*mol/GlcN*	*nmol/mg*	*mol/GlcN*
Sugars								
Abequose	882	8.6	335	0.8	9	0	669	3.0
Rhamnose	857	8.4	325	0.8	0	0	656	2.8
Mannose	808	7.9	307	0.7	0	0	663	2.8
Galactose	1159	11.4	700	1.6	359	0.4	1062	4.5
Glucose	193	1.9	552	1.3	400	0.5	232	1.0
Heptose	55	0.5	411	1.0	283	0.3	145	0.6
KDO	53	0.5	215	0.5	382	0.5	104	0.4
Glucosamine	102	1.0	432	1.0	810	1.0	236	1.0
*Fatty acids**								
12:0	23	0.2	130	0.3	251	0.3	51	0.2
14:0	13	0.1	102	0.2	301	0.4	21	0.1
16:0	10	0.1	75	0.2	125	0.2	20	0.1
2-OH-14:0	10	0.1	45	0.1	35	0.1	37	0.2
3-OH-14:0	95	0.9	557	1.3	1247	1.5	281	1.2
Phosphate	156	1.5	604	1.4	1054	1.3	349	1.5

* 12:0, 14:0. 16:0 are dodeca-, tetradeca- and hexadecanoic acid; 2-OH- and 3-OH- indicate 2- and 3-hydroxy.

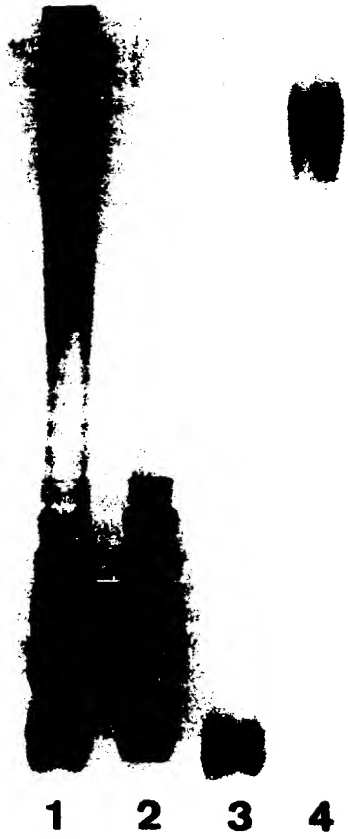

Fig. 1. SDS-PAGE of original *S. abortus equi* lipopolysaccharide and the fractions. 1 = Original LPS; 2 = short-chain fraction; 3 = R-fraction; 4 = long-chain fraction.

SDS-PAGE analysis

The electrophoretic pattern of the fractions in SDS-PAGE is shown in Fig. 1. Long-chain LPS consisted of molecules with 20–50 repeating units in the O-polysaccharide part. This value is obtained assuming that each successive band slower than the R-form band denotes an additional repeating unit in the molecule. It was free of medium-chain-length material and of R-form LPS. The short-chain fraction contained R-LPS and LPS with 1–6 repeating units. The R-fraction was devoid of O-polysaccharide.

Chemical analysis

The results of chemical analysis are shown in Table I. Sugar analysis revealed the presence of O-specific sugars in the long- and short-chain fractions and their absence in the R-fraction. Abequose, mannose and rhamnose are present in a molar ratio of *ca.* 1:1:1. The average molar ratio of each to glucosamine is 8.3:1 in the long-chain and 0.8:1 in the short-chain fraction. Since these sugars are present ex-

clusively in the O-polysaccharide, an average number of *ca.* 25 and 2.5 repeating units per molecule of long- and short-chain preparation, respectively, could be estimated, assuming three glucosamine residues (two in lipid A and 1 in the core) to be present per molecule of LPS.

All three fractions contained glucosamine, heptose and KDO in a molar ratio more or less similar to that in which they are present in the starting *S. abortus equi* LPS. Also the fatty acid content of the fractions is in good correspondence to the high or low amounts of sugars they contain. The long-chain fraction contained the lowest proportion of total fatty acids (0.15 μmol/mg) compared with the starting LPS (0.41 μmol/mg). In the short-chain fraction the concentration of fatty acids was higher than in the starting LPS (0.92 μmol/mg), and highest amounts of fatty acids were present in the R-fraction (1.96 μmol/mg).

Antigenic specificity

The serological characterization of the three fractions revealed that their antigenic specificity is in good agreement with their chemical composition. In the passive haemolysis and passive haemolysis inhibition test, the long-chain fraction reacted strongly with or inhibited anti-*S. abortus equi* O-antibodies. It showed, however, no cross-reaction with antibodies to any of the R classes (Ra to Re).

The short-chain fraction (also containing R-LPS) showed a strong cross-reaction with anti-O-antibodies and also with antibodies to Ra and Rb, but no reaction with antibodies to Rc to Re.

In contrast, the R-fraction was completely devoid of O-antigenicity. It exhibited a strong reaction with anti-Rb and a weaker reaction with anti-Ra antiserum, but no reaction with anti-Rc, -Rd and -Re.

DISCUSSION

Fractionation of the S-form LPS was based on the fact that the length of the O-chains determines the degree of lipophilicity of the molecules. Hence, the solvent mixtures that were used for sequential extraction consisted of organic solvents and increasing amounts of hydrochloric acid. These mixtures were monophasic, but small amounts of water or solutes caused separation into a lower organic phase, which contained preferentially the hydrophobic short-chain and R-LPS, and a hydrophilic upper phase with the long-chain LPS.

The success of the fractionation procedure was examined by SDS-PAGE. The only homogeneous fraction in this test seemed to be the R-LPS (yield 10% of original LPS), which was represented by one band. The short-chain fraction (50%) represented a mixture of R-LPS and LPS containing 1–6 repeating units. The number of repeating units is calculated from the numeric position of the band and by assuming that the molecular mass of bands slower than R-form material increases successively by one repeating unit. The long-chain fraction (30%), which is also heterogeneous, is devoid of R-LPS and consists of molecules containing between 20 and 50 repeating units.

The results of chemical analysis confirm the nature of the three fractions as being rough, and as containing long and short O-polysaccharide chains, respectively (Table II). Thus the R-fraction was devoid of mannose, rhamnose and abequose

TABLE II

RELATIVE MOLAR RATIOS IN THE LIPOPOLYSACCHARIDE AND THE SUBFRACTIONS OF O-SPECIFIC SUGARS AND KDO

Values based on 1 mol glucosamine. O-specific sugars are mannose, rhamnose, abequose, and part of galactose.

Fraction	*O-sugars*	*KDO*
Long-chain fraction	34	0.5
Short-chain fraction	3	0.5
R-fraction	0	0.5
Original lipopolysaccharide	11	0.4

which, together with galactose constitute the specific sugars of the *S. abortus equi* O-antigen. The small amount of galactose found here (6.5%) was to be expected since this sugar is a constituent of the core-oligosaccharide of *Salmonella* LPS. The short- and long-chain fractions contained large amounts of O-specific sugars, the latter in a significantly higher concentration. All three fractions contained the expected core and lipid A constituents, glucose, galactose, heptose, KDO, glucosamine, phosphorus and fatty acids. Their proportions were highest in the R-fraction and lowest in the long-chain fraction.

The antigenic properties of the three fractions are in compiete agreement with their chemical composition. O-antigenic reactivity was present only in the long- and short-chain fractions and was absent from the R-fraction. R-antigenic reactivity was expressed by the R-fraction and the short-chain fraction (which contains R-LPS), but not by the long-chain fraction. This indicates that the core-region in the long-chain LPS is not accessible to R antibodies. It seems therefore that cross-reactions reported frequently to occur between anti-R antibodies and S-form LPS or bacteria are due to variable amounts of R-form (and possibly short-chain LPS) present in S preparations or on the surface of the bacterial cells.

The biological properties of subfractions of S-form LPS are under study. It has been shown already that the activity of S-form LPS to induce granulocyte chemiluminescence is a property of the R-form LPS it contains, and that the long-chain fraction is completely devoid of this activity[11].

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Chemical, Biological, and Immunochemical Properties of the *Chlamydia psittaci* Lipopolysaccharide

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The lipopolysaccharide (LPS) of *Chlamydia psittaci* was extracted from yolk sac-grown elementary bodies, purified, and characterized chemically, immunochemically, and biologically. The LPS contained D-galactosamine, D-glucosamine, phosphorus, long-chain fatty acids, and 3-deoxy-D-*manno*-2-octulosonic acid in the molar ratio of approximately 1:2:2:6:5. The antigenic properties of the isolated LPS were compared with those of the LPS from *Chlamydia trachomatis* and *Salmonella minnesota* Re by the passive hemolysis and passive hemolysis inhibition tests, absorption, hydrolysis kinetics, and Western blot analysis with rabbit polyclonal antisera against chlamydiae and with a mouse monoclonal antibody recognizing a genus-specific epitope of chlamydial LPS. Two antigenic determinants were identified, one of which was chlamydia specific and the other of which was cross-reactive with Re LPS. Both determinants were destroyed during acid hydrolysis, whereby a third antigen specificity was exposed which was indistinguishable from the lipid A antigenicity. In rabbit polyclonal antisera prepared against Formalin-killed elementary bodies or detergent-solubilized membranes, two antibody specificities were differentiated. One of these was chlamydia specific, and the other was cross-reactive with Re LPS. The LPS of *C. psittaci* was inactive within typical endotoxin parameters (lethal toxicity, pyrogenicity, local Shwartzman reactivity); it was, however, active in some in vitro assays, such as those testing for mouse B-cell mitogenicity and the induction of prostaglandin E_2 in mouse peritoneal macrophages.

Chlamydiae are pathogenic, obligatory intracellular parasites causing a variety of diseases in animals and humans (22, 35, 37). Chlamydiae have a unique developmental cycle which includes metabolically active, noninfectious, and multiplying reticulate bodies and metabolically inactive but infectious elementary bodies (35). Surface components of these unique bacteria have been assumed to participate in the early stages of infection (adhesion and penetration) and may be responsible for the inhibition of phagosome-lysosome fusion, which is characteristic of chlamydial infection (10, 17).

These surface components of chlamydiae also represent antigens among which genus-, species-, and subspecies-specific antigens have been found, giving rise to the induction of specific antibodies during infection. Although there is c nsiderable knowledge of the serological and biological properties of these antigens (3, 9, 11–16, 27, 29–31, 36), little is known of the chemistry of these structures. One of the major antigens is a heat-stable, genus-specific glycolipid antigen which has been proposed to be similar to the lipopolysaccharide (LPS) of gram-negative bacteria (9, 11, 15, 16). Recently, we have definitely shown by chemical means that the glycolipid of *Chlamydia trachomatis* serotype L2 is in fact a typical LPS (30), since it contains characteristic structural elements such as D-glucosamine, 3-deoxy-D-*manno*-2-octulosonic acid (KDO), and 3-hydroxy long-chain fatty acids, components which have been detected in all LPS investigated so far (32, 33). Further evidence for the similarity between chlamydial and enterobacterial LPS was btained from serol gical investigations showing a cross-reacti n of chlamydial with enterobacterial LPS of the Re chemotype (9, 11, 29, 31) and with LPS from *Acinetobacter calcoaceticus* (3, 31).

Recently, Caldwell and Hitchcock have reported on a monoclonal antibody which recognizes a genus-specific epitope on the chlamydial LPS which does not react with Re LPS in Western blot analysis (11). Thus, the chlamydial LPS expresses at least two antigenic determinants, one of which is *Chlamydia* specific and the other of which is similar to the Re antigenic determinant. We have confirmed these results with polyclonal antisera against chlamydial and Re-type LPS in a passive hemolysis assay and, moreover, have demonstrated by absorption experiments the presence of two antibody specificities in polyclonal antisera against chlamydiae (9). One of these antibodies reacted with both chlamydial and Re LPS; the other was *Chlamydia* specific. In addition, we have shown that chlamydial LPS contains the lipid A antigenic determinant which, as in other bacteria, is cryptic in LPS and exposed only after acid hydrolysis.

The present study was performed with the aim to further define these antigenic determinants on a molecular level in chemical terms and to gain insight into the structure and the biological functions of chlamydial LPS. We now report on the chemical composition and the biological (endotoxic) and immunochemical properties of the LPS of *Chlamydia psittaci*, one of two species of the genus *Chlamydia*.

MATERIALS AND METHODS

Bacteria and bacterial LPS. *C. psittaci* PK 5082, associated with enzootic abortion in ewes, was propagated in embryonated eggs as previously described (34). Infected yolk sacs from 600 eggs were suspended in 1 M potassium chloride (20%, vol/v l) and inactivated with 0.5% Formalin, followed by high-speed centrifugation (20,000 × *g* for 40 min). The

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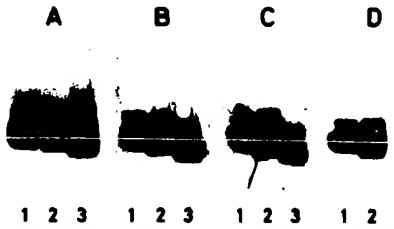

FIG. 1. SDS-PAGE pattern of LPS from *C. psittaci* (lanes 1), *C. trachomatis* (lanes 2), and *S. minnesota* Re (lanes 3). Gels were stained with alkaline silver nitrate (A) or after Western blot transfer, with rabbit polyclonal antiserum against *S. minnesota* Re (B), polyclonal antiserum (absorbed with Re LPS-OH) against chlamydiae (C), or monoclonal antibody L2I–6 against a genus-specific epitope of chlamydial LPS (D).

sediment was suspended in 500 ml of phosphate-buffered saline and digested with trypsin at 37°C for 1 h with stirring. After the addition of isotonic saline (800 ml), the suspension was extracted with an excess of ethyl ether in a separating funnel. The aqueous phase was centrifuged as before, and the sediment was suspended in distilled water, dialyzed against distilled water, and lyophilized. The yield of purified elementary bodies was 640 mg. LPS was extracted by the phenol-chloroform-petroleum ether method (20) as modified recently (30). The LPS was purified by repeated ultracentrifugation and converted to the uniform triethylammonium salt after electrodialysis (18). LPS from *C. trachomatis* serotype L2 (30), *Salmonella abortusequi* (21), *Salmonella minnesota* R595 (Re chemotype) (26), *A. calcoaceticus* NCTC 10305 London (4), and bisphosphorylated lipid A from *Escherichia coli* F515 Re chemotype (7) has been previously described. De-*O*-acylated LPS (LPS-OH) was prepared with sodium methylate (0.5 M, 37°C for 16 h), precipitated and washed with ethanol, and dissolved in water (40).

Chemical analysis. Hexosamines were identified by the Morgan-Elson reaction (38) and by an amino acid analyzer. Phosphorus and KDO levels were determined by the methods of Lowry et al. (25) and Brade et al. (6), respectively. KDO was also determined by combined gas-liquid chromatography-mass spectrometry (GLC-MS) of the carbonyl-reduced and permethylated derivative in comparison with authentic KDO (2). Fatty acids were identified by their methyl esters by GLC-MS after release from LPS by acid hydrolysis (4 M HCl at 100°C for 6 h, followed by 2 M hydrochloride-methanol at 85°C for 8 h) as previously described (40). For quantitative estimations, 3-hydroxytetradecanoic acid and heptadecanoic acid were used as internal standards (both fatty acids were shown not to be present in the LPS).

Antibodies. Monoclonal antibody against the chlamydia-specific epitope was kindly provided by H. D. Caldwell (Rocky Mountain Laboratory, Hamilton, Mont.); it recognizes a genus-specific epitope on chlamydial LPS and is designated L2I–6 (11). Polyclonal antisera against chlamydiae were prepared in rabbits as follows. New Zealand White rabbits were injected intravenously with Formalin-killed *C. psittaci* elementary bodies (50, 100, 100, and 200 μg) on days 0, 4, 7, and 11, respectively, and were bled on day 16. For immunization with *C. trachomatis*, cell walls (kindly provided by M. Nurminen, National Public Health Institute, Helsinki, Finland), were solubilized with detergent and suspended in phosphate-buffered saline, and 5, 10, 10, and 20 μg were injected by the same immunization protocol as before. Polyclonal antisera against Re LPS and lipid A were prepared as already reported (7, 26). The described hemolytic system of normal mouse serum and *Acinetobacter* LPS was used t determine the common LPS determinant which is present in all LPS containing KDO, except those of the Re-type (5). This determinant binds to 28-kilodalton normal mouse serum protein, followed by the activation of complement (4). Antisera were absorbed with sheep erythrocytes (SRBC) and stored at −20°C.

Serological methods. Antibodies were determined in microtiter plates by the passive hemolysis test (7–9) with guinea pig complement, pretested for the absence of chlamydial antibodies and absorbed with SRBC. Inhibition studies, absorption experiments, and hydrolysis kinetics were performed as previously reported (9).

SDS-PAGE. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of LPS was carried out by the method of Laemmli (24) with a 16% gel. Gels were stained with silver nitrate (39) and, after Western blot transfer, with antiserum and monoclonal antibody. Briefly, gels were transferred electrophoretically to nitrocellulose (1), incubated with blocking buffer followed by incubation with mouse monoclonal antibody or with rabbit polyclonal antiserum. The former reacted with goat anti-mouse immunoglobulin G–mouse peroxidase-anti-peroxidase complex, and the latter reacted with peroxidase-labeled goat anti-rabbit immunoglobulin M with 3,3'-diaminobenzidine or 4-chloro-1-naphthol and H_2O_2 as substrates.

Biological assays. Tests were performed to determine lethal toxicity in D-galactosamine-sensitized C57BL/6 mice, pyrogenicity in chinchilla rabbits, local Shwartzman reactivity in New Zealand White rabbits, induction of prostaglandin E_2 (PGE_2) in mouse (NMRI) peritoneal macrophages, B-cell mitogenicity in spleen cells of C3H/Tif and C3H/HeJ mice, and anticomplementary activity in vitro as reported previously (19).

RESULTS

Extraction and purification of *C. psittaci* LPS. The LPS of *C. psittaci* was extracted from purified yolk sac-grown elementary bodies by a modified phenol-chloroform-petroleum ether method with a yield of 1.8% (dry weight). The LPS was purified by repeated ultracentrifugation and converted to uniform triethylammonium salt after electrodialysis. After SDS-PAGE followed by staining with alkaline silver nitrate, one main band was visualized which migrated slightly slower than *C. trachomatis* LPS, which in turn migrated slower than *S. minnesota* Re LPS (Fig. 1A). The absence of nucleic acid was confirmed by UV spectroscopy and by sugar analysis (absence of ribose). The absence of phospholipids was ascertained by the lack of unsaturated

TABLE 1. Chemical composition of the LPS from *C. psittaci*

Compound	Amt present	
	nmol/mg	μg/mg
Glucosamine	513	92
Galactosamine	257	46
Phosphate	578	55
Fatty acids	1,507	443
KDO	1,190	283

fatty acids, namely hexa- and octadecen ic acid. The protein content was calculated by amino acid analysis to be bel w 1%.

Chemical analysis. Chemical analysis revealed the presence of galactosamine, glucosamine, phosphate, fatty acids, and KDO in the molar ratio of approximately 1:2:2:6:5 (Table 1). The identity of KDO was ascertained by GLC-MS f the carbonyl-reduced and permethylated derivatives. The sample under investigation exhibited the same retention time by GLC and superimposable mass spectra after electron impact and chemical ionization mass spectrometry compared with similarly derivatized authentic KDO (2). The quantitative determination of KDO by the thiobarbituric acid assay yielded 1,190 nmol of LPS per mg, corresponding to 28.3%. When the thiobarbiturate assay was performed without hydrolysis before periodate oxidation, 520 nmol/mg was found, indicating that approximately one half of the total KDO was substituted at position 4 or 5 or both (6). With different hydrolysis conditions for the liberation of KDO from LPS (6), the same values were obtained after hydrolysis in either acetate buffer or 0.1 M hydrochloric acid. indicating that the aforementioned substituent was present in a very acid-labile linkage.

Fatty acids were detected and identified by combined GLC-MS of the methyl esters (1,507 nmol per mg of LPS), whereby straight-chain and branched and nonhydroxylated and 3-hydroxylated fatty acids were found (Table 2). The latter exhibited an unusual length of the hydrocarbon chain, with 18 to 22 carbon atoms. In sodium methylate-treated LPS (with ester-linked fatty acids removed), only 3-hydroxy fatty acids were detected.

Immunochemical properties. The immunogenicity of chlamydial LPS was investigated with rabbits with solubilized membranes of *C. trachomatis* elementary bodies and Formalin-killed elementary bodies of *C. psittaci*. The animals (eight for each immunogen) were shown to have no preimmunization titers against either chlamydial or Re LPS. A short-term immunization protocol (four intravenous injecti ns at 4-day intervals) resulted in high hemolytic antibody titers against both chlamydial and Re LPS. Chlamydial antibody titers, however, were about 10-fold higher than those measured against Re LPS and reached values of up to 40,000 (individual titers are not listed; representative results are included in Tables 3 and 4).

SRBC were sensitized with various amounts of LPS-OH of *C. trachomatis*, *C. psittaci*, and *S. minnesota* Re and used in the passive hemolysis assay with homologous and heterologous antisera. The results shown in Table 3 are representative for all antisera used. With only 4 μg of LPS-OH per 0.2 ml of packed SRBC, a significant sensitization was observed; however, 40 to 80 μg was required for an optimal sensitization, as indicated by a plateau value of the antibody titers. For further experiments, 80 μg of LPS-OH per 0.2 ml of SRBC was used.

The antisera were absorbed with LPS-OH of *S. minnesota* Re and tested against the homologous and heterologous antigens (Table 4). In antisera against *C. psittaci* and *C. trachomatis*, the titer against Re LPS-OH was eliminated whereas the titer against the LPS of both chlamydial species was not reduced by absorption. Absorption of the Re antiserum with the homologous antigen completely eliminated

TABLE 2. Fatty acid composition of the LPS from *C. psittaci*

Fatty acid	Amt present	
	nmol/mg	μg/mg
$C_{14:0}$	74	17
$C_{15:0}$ br[a]	106	26
$C_{16:0}$ br	81	21
$C_{16:0}$	254	64
$C_{18:0}$ br	95	27
$C_{18:0}$	113	32
$C_{18:0}$(3-OH)	Trace[b]	Trace
$C_{20:0}$ br	120	36
$C_{20:0}$	95	29
$C_{20:0}$(3-OH)	309	101
$C_{20:0}$(3-OH) br	37	12
$C_{21:0}$(3-OH) br	223	76
$C_{22:0}$(3-OH) br	Trace	Trace

[a] br, Branched.
[b] <10 nmol/mg.

TABLE 3. Reactivity of anti-*C. trachomatis*, anti-*C. psittaci*, and anti-Re antisera with SRBC coated with chlamydial or Re LPS-OH

Sensitizing antigen (amt, μg/0.2 ml of SRBC)	Hemolytic titer with antiserum against:		
	C. trachomatis	*C. psittaci*	*S. minnesota* Re
C. trachomatis LPS-OH			
4	320	640	160
8	5,120	10,240	160
40	20,480	20,480	640
80	20,480	20,480	1,280
200	20,480	20,480	1,280
C. psittaci LPS-OH			
4	1,280	10,240	320
8	10,240	40,960	640
40	10,240	40,960	1,280
80	10,240	40,960	1,280
200	10,240	40,960	1,280
S. minnesota Re LPS-OH			
4	80	80	40
8	160	160	160
40	1,280	1,280	1,280
80	2,560	2,560	2,560
200	2,560	2,560	2,560

TABLE 4. Hemolytic activity of anti-*C. trachomatis*, anti-*C. psittaci*, and anti-Re antisera before and after absorption with Re LPS-OH-coated SRBC[a]

Antiserum before and after absorption	Hemolytic titer against SRBC coated with LPS-OH of:		
	C. trachomatis	*C. psittaci*	*S. minnesota* Re
C. trachomatis			
Before	20,480	20,480	1,280
After	20,480	20,480	<20
C. psittaci			
Before	10,240	40,960	1,280
After[b]	10,240	40,960	<20
S. minnesota Re			
Before	2,560	2,560	2,560
After	<20	<20	<20

[a] SRBC were sensitized with 80 μg of the respective LPS-OH per 0.2 ml of packed cells; 0.5 ml of diluted (1:5) antiserum was absorbed with 50 μl of SRBC sensitized with *S. minnesota* Re LPS-OH.
[b] Same antiserum as the one indicated in Fig. 1C.

TABLE 5. Passive hemolysis inhibition test of chlamydial and Re LPS-OH in homologous and heterologous antigen-antibody systems

Inhibitor LPS-OH	Inhibition value (ng) obtained in the indicated hemolytic system[a]		
	C. trachomatis–anti-*C. trachomatis*	*C. psittaci*–anti-*C. psittaci*	*S. minnesota* Re–anti-*S. minnesota* Re
C. trachomatis	1	32	32
C. psittaci	16	1	63
S. minnesota Re	>1,000	>1,000	16

[a] In the antigen-antibody systems, the respective LPS-OH and 3 hemolytic U of homologous antiserum were used. Values indicate amount inhibiting 50% of lysis.

the hemolytic activity against the homologous (Re) and the heterologous (chlamydial) LPS antigens.

The chlamydial and Re LPS were further characterized by inhibition experiments with the homologous antigen-antibody hemolytic systems of *C. trachomatis*, *C. psittaci*, and *S. minnesota* Re (Table 5). For *C. trachomatis* and *C. psittaci*, 1 ng of LPS inhibited their respective homologous systems; for the Re system, 32 and 63 ng, respectively, were inhibitory. The Re LPS inhibited only the homologous antigen-antibody system, yielding a 50% inhibition value of 16 ng, and did not inhibit either chlamydial system in amounts of up to 1 μg.

The behavior of different antigenic specificities of the chlamydial LPS after acid hydrolysis was monitored kinetically. In addition to the aforementioned test systems, lipid A–anti-lipid A was used to detect the lipid A antigenic determinant (Fig. 2). The *Chlamydia*- and Re-specific determinants were destroyed during hydrolysis in both chlamydial LPS, as indicated by a drastic decrease of the inhibitory capacity of the respective antigen-antibody system. After 60 min of hydrolysis, neither LPS inhibited the Re and chlamydial antisera any longer. The specificity of the hemolytic system for lipid A antigenicity was not inhibited by intact LPS (time zero in Fig. 2). Lipid A antigenicity was, however, exposed during hydrolysis and reached a maximum after 15 (*C. psittaci*) and 30 (*C. trachomatis*) min, with inhibition values of 63 and 16 ng, respectively. Neither the intact LPS nor the hydrolyzed samples inhibited the reaction of normal mouse serum with *Acinetobacter* LPS, which is specific for a common determinant containing KDO and neutral sugar (8) and recognized by a protein of normal mouse serum (5).

The LPS were also characterized by SDS-PAGE, followed by staining with silver nitrate (Fig. 1A) or, after Western blot transfer, with antisera (Fig. 1B and C) or monoclonal antibody (Fig. 1D). The LPS of *C. psittaci* (lane 1), *C. trachomatis* (lane 2), and *S. minnesota* Re (lane 3) each exhibited one major band with a slightly different migration pattern with the Re LPS moving faster than the LPS of both chlamydial preparations and the *C. trachomatis* LPS migrating faster than the *C. psittaci* LPS. Both chlamydial LPS stained with monoclonal antibody L2I–6 (Fig. 1D) and with polyclonal anti-Re antiserum (Fig. 1B). They were also visualized with polyclonal antiserum against *C. psittaci* which had been absorbed with Re LPS-OH (Fig. 1C). The Re LPS was not detected by the monoclonal antibody against chlamydial LPS (Fig. 1D, lane 3) but stained as expected with polyclonal Re antiserum (Fig. 1B, lane 3). The Re-absorbed antiserum against *C. psittaci* yielded a faint staining of the R LPS (Fig. 1C, lane 3), indicating that the adsorption had not been quantitative.

Biological activities. The LPS of *C. psittaci* was tested in a number of in vivo and in vitro assays for endotoxic and other biological activities for comparison with a well-characterized and standardized endotoxin from *S. abortusequi* (21). The chlamydial LPS was not pyrogenic for rabbits (5 μg/kg), was not toxic for galactosamine-sensitized mice (10 μg per mouse), and did not induce a local Shwartzman-reaction at 50 μg each for the intradermal preparative and the intravenous challenge doses. The same held true for animals which were prepared intradermally by the LPS of *S. abortusequi* and which were challenged intravenously with *C. psittaci* LPS and vice versa with the same dosage as before. The chlamydial LPS also failed to activate guinea pig complement in amounts of up to 50 μg. The LPS was, however, active in in vitro assays for mouse B-cell mitogenicity and PGE_2 induction in mouse peritoneal macrophages. The mitogenic activity of *C. psittaci* LPS for responsive (C3H/Tif) and nonresponsive mice (C3H/HeJ) is illustrated in Fig. 3. The LPS induced a typical dose-dependent response in the splenocytes of responder mice, with a maximum of 10 μg. The curve was comparable to the ones reported for the LPS of *S. abortusequi* (21) and the synthetic *Escherichia coli* lipid A (19). Although nonresponder mice gave a lower response (closed circles in Fig. 3), the uptake of thymidine was significantly elevated over background values. The induction of PGE_2 in mouse peritoneal macrophages is shown in Table 6. The LPS of *C. psittaci* yielded values comparable to those of the standard endotoxin.

DISCUSSION

The LPS of *C. psittaci* was extracted from yolk sac-grown elementary bodies purified and characterized chemically, immunochemically, and biologically. The results were compared with those published recently for the LPS of *C. trachomatis* (9, 30).

The yield of *C. psittaci* LPS was 1.8% (dry weight) of elementary bodies, which is comparable to that reported for *C. trachomatis* LPS (30). Chemical analysis revealed the presence of D-glucosamine, KDO, 3-hydroxy long chain

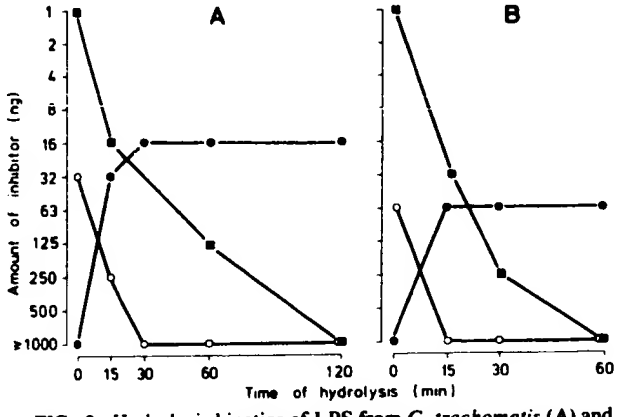

FIG. 2. Hydrolysis kinetics of LPS from *C. trachomatis* (A) and *C. psittaci* (B) in acetate buffer (0.1 M [pH 4.4] at 100°C). Samples were tested by the passive hemolysis inhibition test with the antigen-antibody systems of Re LPS-OH–anti-Re (○), *C. psittaci* LPS-OH–anti-*C. psittaci* (■), and lipid A-OH–anti-lipid A (●).

fatty acids, and phosphate, constituents which are typical for all LPS investigated so far (32, 33) and which have been also detected in *C. trachomatis* (30). In addition, D-galactosamine was identified as a second hexosamine, which had not been found in *C. trachomatis*. Further investigations will answer the question of whether this amino sugar is a species- or a strain-specific component of *C. psittaci*. Another chemical difference between the LPS of *C. psittaci* and *C. trachomatis* is the amount of KDO present. Whereas 28% of the former was made up of this common LPS component, 20% was estimated for the latter (30). As found with *C. trachomatis* LPS, the fatty acid pattern of *C. psittaci* was characterized by unusual 3-hydroxy fatty acids with 18 to 22 carbon atoms and by a number of nonhydroxylated fatty acids among which iso- and anteiso-branched fatty acids were found. (In a previous paper, 3-hydroxy $C_{21:0}$ branched fatty acid was erroneously reported as $C_{20:0}$ [30].) All constituents analyzed accounted for 92% of gross weight, indicating that the results were representative for the sample under investigation. Major contaminations of the LPS with protein, nucleic acids, or phospholipids were excluded, and relative homogeneity in size was shown by SDS-PAGE, followed by silver staining. It should be noted, however, that this does not exclude microheterogeneity, which can be expected from the acylation pattern and which is known for all LPS (28). During SDS-PAGE, it was also seen that the LPS of *C. trachomatis* migrated slightly faster than that of *C. psittaci*, indicating a higher molecular weight of the latter. Both chlamydial LPS were larger than the Re LPS of *S. minnesota*.

The immunogenic and antigenic properties of *C. psittaci* LPS were investigated by immunizing rabbits with either Formalin-killed *C. psittaci* elementary bodies or detergent-solubilized *C. trachomatis* membranes. With a simple, short-term immunization protocol (leading to the production of immunoglobulin M-rich immune sera), all animals were found to respond with high antibody titers to the immunogen. These antisera were characterized by the passive hemolysis and passive hemolysis inhibition assays, absorption experiments, hydrolysis kinetics, and Western blot analysis with isolated chlamydial LPS of both species as an antigen. A monoclonal antibody against a genus-specific epitope of chlamydial LPS (11) and the Re-specific antigen-antibody system were included in these experiments. Polyclonal antisera raised against *C. trachomatis* or *C. psittaci* were found to contain at least two different antibody specificities; one of these antibodies reacted with both chlamydial and Re-type LPS and the other was chlamydia specific. This was shown by absorption, whereby the former could be absorbed with Re LPS, which did not affect the latter. After absorption with Re LPS, the anti-chlamydiae antisera exhibited the same specificity as that of the monoclonal antibody in Western blot analysis.

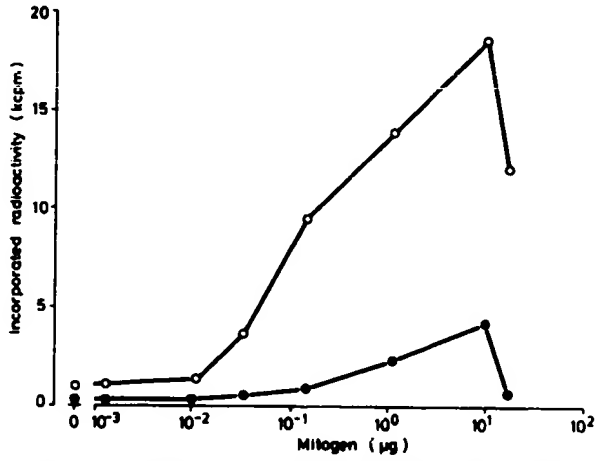

FIG. 3. Mitogenicity of *C. psittaci* LPS for spleen cells from C3H/Tif (O) and C3H/HeJ ( ).

TABLE 6. Release of PGE_2 from mouse peritoneal macrophages upon stimulation with LPS from *C. psittaci* and *S. abortusequi*

LPS	Amt (µg) of LPS	PGE_2 (ng) released
None (control)	0	2.1
C. psittaci	1	33.8
	10	37.2
S. abortusequi	1	24.8
	10	27.4

The LPS of *C. psittaci* was found to contain at least two different antigenic determinants. This was proven in inhibition studies with monospecific antigen-antibody systems. One of these determinants was shared by chlamydial and Re-type LPS, yielding comparable inhibition values in the Re-specific system, whereas the second determinant inhibited only the chlamydial antigen-antibody reaction. For both *C. psittaci* and *C. trachomatis* 1 ng of LPS inhibited their respective homologous systems whereas signficantly higher inhibition values (16 and 32 ng, respectively) were found in the heterologous reaction (Table 5). We tentatively propose that *C. psittaci* possesses, in addition to the genus-specific epitope, a second, species-specific determinant located in the carbohydrate moiety of LPS. A chemical substrate for this antigenic determinant could be seen in the presence of D-galactosamine in the LPS of *C. psittaci*.

Hydrolysis kinetics performed on both LPS showed that the antigen specificities mentioned above were destroyed during hydrolysis; however, exposure was observed of the lipid A antigenic determinant which was indistinguishable from the specificity of bisphosphorylated lipid A (7). In that respect, the chlamydial LPS behaved like all other LPS investigated; lipid A antigenicity and immunogenicity were cryptic in LPS and exposed after acid hydrolysis.

Finally, the biological activities of *C. psittaci* LPS were investigated in vivo and in vitro. The LPS was not active by classical endotoxin parameters, *e.g.*, lethal toxicity, pyrogenicity, or local Shwartzman reactivity (21); it was, however, active in some in vitro assays, such as mitogenic stimulation of mouse B cells and the activation of peritoneal macrophages to produce PGE_2. The low endotoxic activity of this LPS cannot be explained on a molecular basis, since the amounts required for a structural analysis of the lipid A component (which represents the endotoxic principle of LPS [19]) are not available. The activity level may be attributed to the unusual acylation pattern, which certainly enhances the hydrophobicity of this LPS. It can be imagined that the low endotoxic activity is a result of the evolutionary adaptation of chlamydiae to intracellular persistence in host cells (35). In this respect, it is stressed that despite the lack of DNA homology between the two chlamydial species (23), their LPS have been conserved in a similar way.

In summary, this study shows that chlamydial LPS exhibits properties which are shared by other LPS, as well as those properties unique to these obligate intracellular pathogens.

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